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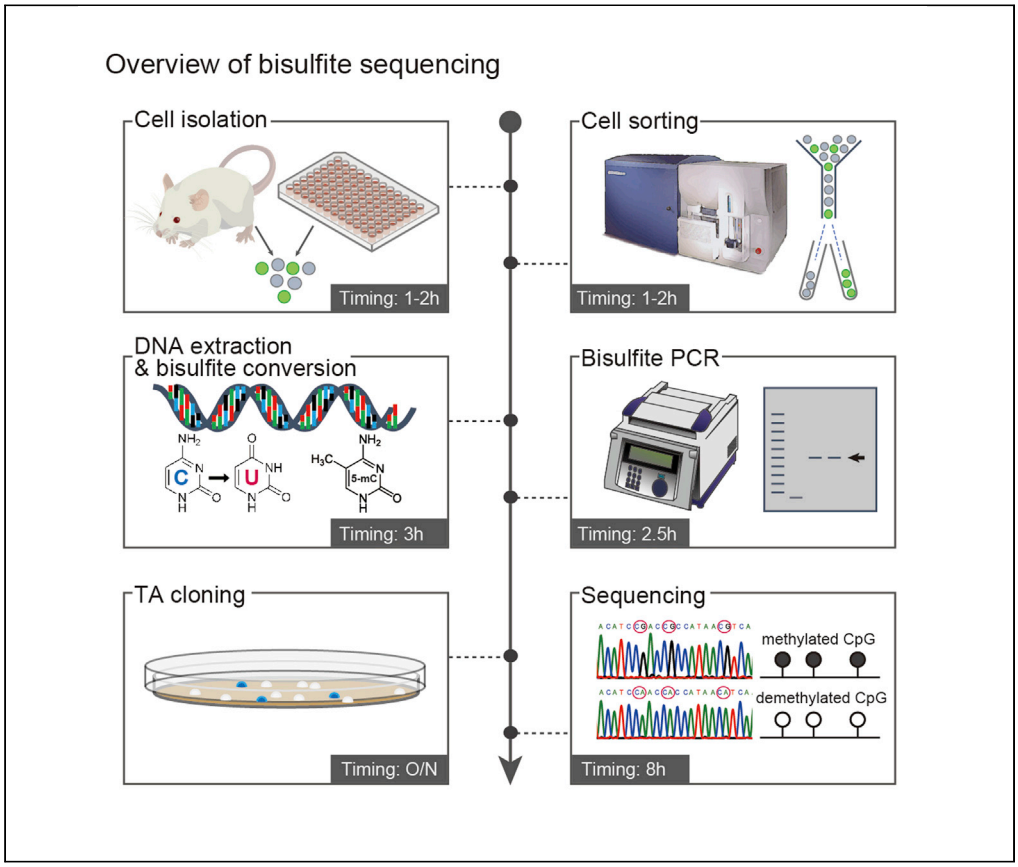
Protocol

Protocol to evaluate cell lineage stability of mouse natural and induced regulatory T cells using bisulfite sequencing



The establishment of regulatory T cells (Treg)-specific demethylation regions (TSDRs) is essential for the Treg-linage stability. Here, we present a protocol using bisulfite sequencing to assess Treg-lineage stability. The protocol describes the isolation of lymphocytes and DNA extraction, followed by bisulfite conversion in unmethylated CpG DNA, bisulfite PCR and cloning, and sequencing to define the TSDR methylation. This protocol uses lymph nodes and spleen tissues and can be adapted to assess the methylation status of Tregs in other tissue types.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Bisulfite sequencing of Treg-specific demethylation regions (TSDR)

Cell isolation, antibody staining, and sorting of CD4+ T cells

Detailed procedures for bisulfite conversion of unmodified cytosine into uracil

Methylation status in TSDR indicates the stability of Treg cell-lineage

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Protocol to evaluate cell lineage stability of mouse natural and induced regulatory T cells using bisulfite sequencing

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SUMMARY

The establishment of regulatory T cells (Treg)-specific demethylation regions (TSDRs) is essential for the Treg-lineage stability. Here, we present a protocol using bisulfite sequencing to assess Treg-lineage stability. The protocol describes the isolation of lymphocytes and DNA extraction, followed by bisulfite conversion in unmethylated CpG DNA, bisulfite PCR and cloning, and sequencing to define the TSDR methylation. This protocol uses lymph nodes and spleen tissues and can be adapted to assess the methylation status of Tregs in other tissue types.

BEFORE YOU BEGIN

The protocol below describes the specific steps to investigate the methylation status in TSDRs by bisulfite sequencing, and focuses on the isolation of T cells from lymph nodes and spleen. If it is necessary to assess the methylation status in other tissue-derived Tregs, the same procedure from DNA extraction to the final sequencing can be used.

Institutional permissions

All animals used in this protocol were maintained in specific pathogen-free condition. All animal studies were performed following the guidelines on animal welfare of Osaka University.

Mice

Mouse strain selection will depend on the experiment. In this protocol, we have analyzed 8–12 weeks old BALB/c background mice, as well as various transgenic strains.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD4-BV650 (RM4-5) (dilution used 1:200)	BD Biosciences	Cat#563747; RRID: AB_2716859
Purified anti-mouse CD16/32 (93) (dilution used 1:200)	BioLegend	Cat#101302; RRID: AB_312801
Anti-mouse CD25-BV421 (PC61) (dilution used 1:200)	BioLegend	Cat#102034; RRID: AB_11203373

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-mouse CD25-PE (PC61) (dilution used 1:200)	BD Pharmingen	Cat#553866; RRID: AB_395101
Anti-mouse CD44-APC (IM7) (dilution used 1:200)	Invitrogen	Cat#17-0441-83; RRID: AB_469390
Anti-mouse CD62L-BV421 (MEL-14) (dilution used 1:200)	BD	Cat#562910; RRID: AB_2737885
Anti-mouse CD62L-PerCP-Cy5.5 (MEL-14) (dilution used 1:200)	BD Pharmingen	Cat#560513; RRID: AB_10611578
Anti-mouse Foxp3-Alexa Fluor 488 (FJK-16s) (dilution used 1:200)	Invitrogen	Cat#53-5773-82; RRID: AB_763537
Bacterial and virus strains		
E. coli DH5α Competent Cells	Takara	Cat#9057
Chemicals, peptides, and recombinant proteins		
D-PBS (–) (1×)	Nacalai Tesque	Cat#14249-24
RPMI 1640	Nacalai Tesque	Cat#30264-85
0.5 M EDTA	Nacalai Tesque	Cat#06894-14
Penicillin-streptomycin	Nacalai Tesque	Cat#26253-84
Fetal Bovine Serum (FBS)	Gibco	Cat#10437028 Lot#1183841
2-β-Mercaptoethanol (2-ME)	Gibco	Cat#21985023
2-Phospho-L-ascorbic acid trisodium salt ≥95.0% (A2P)	Sigma-Aldrich	Cat#49752-10G
Tamibarotene	Selleck	Cat#S4260-50mg
UltraPure™ 1 M Tris-HCl Buffer, pH 7.5	Invitrogen	Cat#15567027
Sodium Chloride	Nacalai Tesque	Cat#31319-45
Sodium Lauryl Sulfate (SDS)	Nacalai Tesque	Cat#31606-62
Proteinase K Solution (20 mg/mL), RNA grade	Invitrogen	Cat#25530049
Phenol:Chloroform:Isoamylalchol (PCI)	Nacalai Tesque	Cat#26058-96
Chloroform:Isoamylalcohol (CIA)	Sigma-Aldrich	Cat#C0549-1PT
Sodium Acetate	Nacalai Tesque	Cat#31137-25
Ethachinmate	NIPPON GENE	Cat#318-01793
Ethanol (95) (use it as 100% EtOH)	Nacalai Tesque	Cat#14710-25
NaOH	Wako	Cat#198-13765
Sodium bisulfite	Sigma-Aldrich	Cat#243973
Sodium disulfite	Merck	Cat#106528
Buffer PB (500 mL)	QIAGEN	Cat#19066
Buffer PE (concentrate, 100 mL)	QIAGEN	Cat#19065
CAPS	Dojindo	Cat#347-00482
50 × TAE	NIPPON GENE	Cat#313-90035
Agarose S (500 g)	NIPPON GENE	Cat#318-01195
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Nacalai Tesque	Cat#19742-94
5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)	Nacalai Tesque	Cat#06280-31
Bacto™ Tryptone	BD Biosciences	Cat#211705
Bacto™ Yeast Extract	BD Biosciences	Cat#212750
Bacto™ Agar	BD Biosciences	Cat#214010
LB Broth, Miller	Nacalai Tesque	Cat#20068-75
KCl	Nacalai Tesque	Cat#28514-75
$MgSO_4$	Nacalai Tesque	Cat#21003-75
$MgCl_2$	Nacalai Tesque	Cat#20909-55
Glucose	Wako	Cat#049-31165
Kanamycin Sulfate	Nacalai Tesque	Cat#19860-44
Water	Nacalai Tesque	Cat#06442-95
Critical commercial assays		
Red Blood Cell Lysing Buffer Hybri-Max™	Sigma-Aldrich	Cat#R7757
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	Thermo Fisher Scientific	Cat#L34975

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse CD4 T Lymphocyte Enrichment Cocktail	BD	Cat#51-9000633
Streptavidin Particles Plus	BD	Cat#51-9000810
eBioscience™ Fixation/Permeabilization Concentrate	Invitrogen	Cat#00-5123-43
eBioscience™ Fixation/Permeabilization Diluent	Invitrogen	Cat#00-5223-56
eBioscience™ Permeabilization Buffer (10×)	Invitrogen	Cat#00-8333-56
Methyl Easy Xceed kit (Human Genetic Signatures)	Takara	Cat#ME002
UltraPure™ Salmon Sperm DNA Solution	Invitrogen	Cat#15632011
Zymo-Spin IC Columns	Zymo Research	Cat#C1004-50
Collection Tubes	Zymo Research	Cat#C1001-50
TaKaRa Ex Taq® Hot Start Version	Takara	Cat#RR006A
QIAEX II Gel Extraction Kit (150)	QIAGEN	Cat#20021
DynaExpress TA PCR Cloning Kit	BioDynamics	Cat#DS130L
illustra TempliPhi DNA Amplification Kit	GE Healthcare	Cat#28964286
BigDye™ Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems	Cat#4337454
Agencourt CleanSEQ Dye-Terminator Removal Kit	Beckman Coulter	Cat#A29154
Experimental models: Organisms/strains		
Mice: BALB/c (male 8–12 weeks old)	CLEA Japan	N/A
Mice: Foxp3-eGFP (male 8–12 weeks old)	Sakaguchi Lab	N/A
Oligonucleotides		
Primers for bisulfite PCR (see Table 4)	Ohkura et al. (2012)	See Table 4
Primers for sequencing (see Table 8)	N/A	See Table 8
Software and algorithms		
FlowJo_v10	FlowJo	https://www.flowjo.com/
MethPrimer 2.0	Li and Dahiya (2002)	http://www.urogene.org/methprimer2/
Sequencing Analysis Software Version v6.0	Applied Biosystems	N/A
Other		
Millex-GV Syringe Filter Unit, 0.22 µm, PVDF, 33 mm, gamma sterilized	Millipore	Cat#SLGVR33RS
60 mm / Non-treated Dish	Iwaki	Cat#1010-60
40 or 70 µm Cell Strainer	Corning	Cat#352340 Cat#352350
3 mL Syringes	Nipro	Cat#08-470
BD FACSAria II cell sorter	BD	N/A
BD IMag™ Cell Separation Magnet	BD	Cat#552311
Eppendorf ThermoMixer® C	Eppendorf	Cat#5382000023
Eppendorf SmartBlock™ 1.5 mL	Eppendorf	Cat#5360000038
Block incubator	Astec Co., Ltd.	Cat#BI-516S
Veriti™ 96-Well Thermal Cycler	Applied Biosystems	Cat#43-757-86
EYELA Water bath	Tokyo Rikakikai Co., Ltd.	Cat#NTT-2200
96S Super Magnet Plate	Alpaqua	Cat#A001322
3500×L Genetic Analyzer	Applied Biosystems	N/A

MATERIALS AND EQUIPMENT

FACS buffer

Reagent	Final concentration	Amount
D-PBS (–)	N/A	489.5 mL
0.5 M EDTA	1 mM	500 µL
FBS	2% (v/v)	10 mL
Total	**N/A**	**500 mL**

Store at 4°C for up to 3 months.

Lysis buffer		
Reagent	Final concentration	Amount
5 M NaCl	100 mM	1 mL
1 M TrisHCl	10 mM	500 μL
0.5 M EDTA	50 mM	5 mL
10% SDS	0.5% (v/v)	2.5 mL
Water	N/A	Up to 50 mL
Total	**N/A**	**50 mL**
Store at room temperature (18°C–25°C) for up to 12 months.		

T cell culture medium		
Reagent	Final concentration	Amount
RPMI 1640	N/A	444.5 mL
Penicillin-streptomycin	1% (v/v)	5 mL
FBS	10% (v/v)	50 mL
2-ME	0.1% (v/v)	500 μL
Total	**N/A**	**500 mL**
Store at 4°C for up to 3 months.		

Note: FBS should be sterile filtered through a 0.22 mm filter.

TE buffer		
Reagent	Final concentration	Amount
1 M TrisHCl	10 mM	500 μL
0.5 M EDTA	1 mM	100 μL
Water	N/A	Up to 50 mL
Total	**N/A**	**50 mL**
Store at room temperature for up to 12 months.		

TAE buffer		
Reagent	Final concentration	Amount
50 × TAE	1×	980 mL
Milli-Q water	N/A	20 mL
Total	**N/A**	**1 L**
Store at room temperature for up to 3 months.		

2% agarose gel		
Reagent	Final concentration	Amount
Agarose S	2% (w/v)	8 g
TAE buffer	N/A	400 mL
Total	**N/A**	**400 mL**
Solidify agarose gel and store in TAE buffer at room temperature for up to 6 months.		

LB medium		
Reagent	Final concentration	Amount
BactoTM Tryptone	2% (w/v)	10 g
Bacto Yeast Extract	0.5% (w/v)	2.5 g
5 M NaCl	10 mM	1 mL

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Continued		
Reagent	Final concentration	Amount
1 M KCl	2.5 mM	1.25 mL
Milli-Q water	N/A	Up to 500 mL
Total	**N/A**	**500 mL**

Autoclave 121°C 20 min, then store at 4°C for up to 6 months.

SOC solution		
Reagent	Final concentration	Amount
LB medium	N/A	480 mL
1 M $MgSO_4$	10 mM	5 mL
1 M $MgCl_2$	10 mM	5 mL
1M glucose	20 mM	10 mL
Total	**N/A**	**500 mL**

Store at 4°C for up to 6 months.

Kanamycin-added LB plate		
Reagent	Final concentration	Amount
LB Broth, Miller	2.5% (w/v)	12.5 g
Bacto™ Agar	1.5% (w/v)	7.5 g
Milli-Q water	N/A	500 mL
Total	**N/A**	**500 mL**

Autoclave 121°C 20 min, cool at 50°C, then add 400 μL 50 mg/mL Kanamycin Sulfate to LB medium solution, make plate medium, and store at 4°C for up to 6 months.

STEP-BY-STEP METHOD DETAILS

Sampling and cell isolation

Timing: 1–2 h

This section describes sampling lymph nodes from mice and preparation of cell suspension.

1. Set the 40–70 μm cell strainer on the 6 cm dish, and add 3 mL FACS buffer into them (A) (Figure 1).
2. Collect peripheral lymph nodes and spleen, transfer them to (A). Mash them using 3 mL syringe plunger.
3. Transfer cell suspension into 15 mL tube. Centrifuge the sample at 500 × *g* for 5 min at 4°C.
4. Discard the supernatant and resuspend the sample with 1 mL Red Blood Cell Lysing Buffer Hybri-Max. Set sample for 2 min at room temperature.
5. Add 9 mL FACS buffer. Centrifuge the sample at 500 × *g* for 5 min at 4°C.
6. Discard the supernatant and resuspend the pellet with 100 μL Mouse CD4 T Lymphocyte Enrichment Cocktail and 200 μL FACS buffer. Set the sample for 15 min on ice.
7. Centrifuge the sample at 500 × *g* for 5 min at 4°C.
8. Discard the supernatant and resuspend the pellet with 100 μL Streptavidin Particles Plus and 200 μL FACS buffer. Set the sample for 15 min on ice.
9. Add 2 mL FACS buffer, transfer the sample to 5 mL tube. Set this tube on Cell Separation Magnet for 6 min at room temperature.
10. Transfer the supernatant to 15 mL tube. Centrifuge the sample at 500 × *g* for 5 min at 4°C.
11. Discard the supernatant and resuspend the pellet with 1 mL FACS buffer.

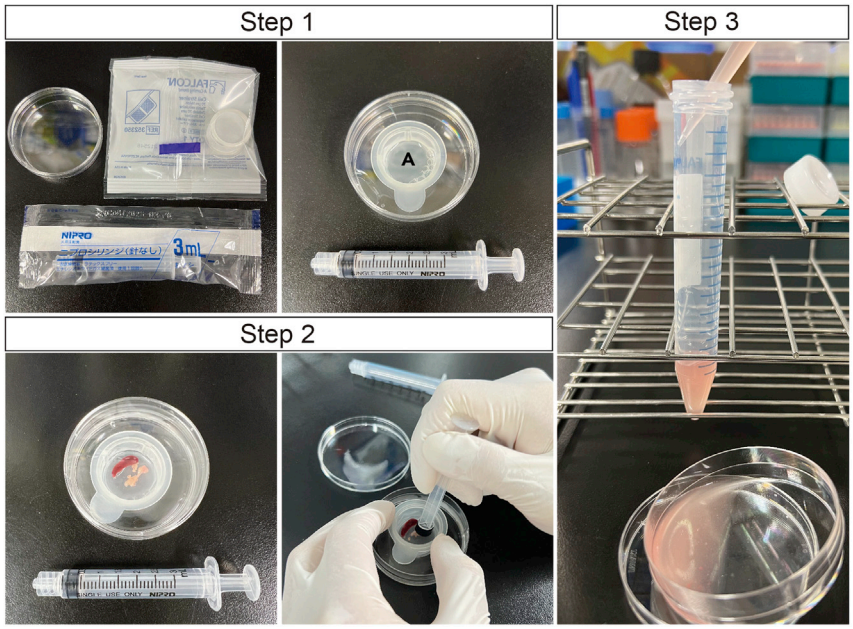

Figure 1. Cell isolation from mouse lymph nodes and spleen

Placing lymph nodes and spleen into a cell strainer (A in Step 1) and mashing them using 3 mL syringe plunger in the 60 mm dish.

Cell staining and sorting

Timing: 1–2 h

This section describes cell staining by antibodies and cell sorting by FACS.

12. Prepare the antibody cocktail below (Table 1).

Note: We prepare 5 times dilution Dead Cell Stain Kit freshly diluted by FACS buffer.

13. Stain the cells with the antibody cocktail. Set the sample for 15 min on ice.
14. Wash the cells with FACS buffer. Centrifuge the sample at 500 × *g* for 5 min at 4°C.
15. Repeat step 14 (if using intracellular staining, move to step a below).

Note: If you use mice without Foxp3 reporter (ex. GFP etc.), you should stain the cells along the protocol below.

Table 1. Antibody cocktail for staining lymphocytes

Reagent	Final concentration	Amount
Purified anti-mouse CD16/32 (93)	1:200	1 μL
Anti-mouse CD4-BV650 (RM4-5)	1:200	1 μL
Anti-mouse CD25-PE (PC61)	1:200	1 μL
Anti-mouse CD44-APC (IM7)	1:200	1 μL
Anti-mouse CD62L-BV421 (MEL-14)	1:200	1 μL
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (About dilution, see the Note below)	1:1000	1 μL
FACS buffer		200 μL

Table 2. Antibody cocktail for intracellular staining lymphocytes

Reagent	Final concentration	Amount
Anti-mouse Foxp3-Alexa Fluor 488 (FJK-16s)	1:200	1 μL
Permeabilization buffer		200 μL

a. Discard the supernatant and resuspend the pellet with 200 μL fixation buffer (Fixation/Permeabilization Concentrate: Fixation/Permeabilization Diluent = 1 : 3). Set the sample for 30 min on ice.
b. Wash the cells with permeabilization buffer. Centrifuge the sample at 600 × *g* for 5 min at 4°C.
c. Repeat step b.
d. Discard the supernatant and resuspend the pellet with the antibody cocktail below (Table 2).
e. Wash the cells with FACS buffer. Centrifuge the sample at 600 × *g* for 5 min at 4°C.
f. Repeat step e.
g. Discard the supernatant and resuspend the pellet with 2 mL FACS buffer. Now, it is ready to sort the cells.

16. Discard the supernatant and resuspend the pellet with 2 mL FACS buffer. Now, it is ready to sort the cells.
17. Sort the cells (up to 100,000 cells each) into 1.5 mL tube with T cell culture medium using FACSAria II cell sorter with the gating strategy below (Figure 2).

Note: For *in vitro* induced Treg (iTreg) cells induction, we referred Mikami et al., (2020), induced and sorted iTreg cells in the same way above.

Cell lysis

Timing: Overnight (12–24 h)

This section describes cell lysis and protein digestion.

18. Centrifuge the sample tube at 500 × *g* for 5 min at 4°C.
19. Discard the supernatant carefully and resuspend the pellet with 400 μL Lysis buffer.
20. Add 2 μL Proteinase K solution to the tube.
21. Shake and incubate the tube at 1,300 rpm for overnight at 55°C.

Note: If you sort the fixed cells, please shake and incubate the tube at 65°C for removing crosslink.

Pause point: If you need the stopping at step 19, store the sample at –30°C (also available from –20°C to –80°C) after resuspension.

DNA extraction

Timing: 1 h

This section describes DNA extraction from cell lysed buffer.

22. Add 400 μL PCI to the sample tube and vortex well. Centrifuge it at 15,000 × *g* for 5 min at room temperature.
23. Transfer the upper layer (400 μL aqueous layer) to the new 1.5 mL tube.
24. Repeat steps 22 and 23.

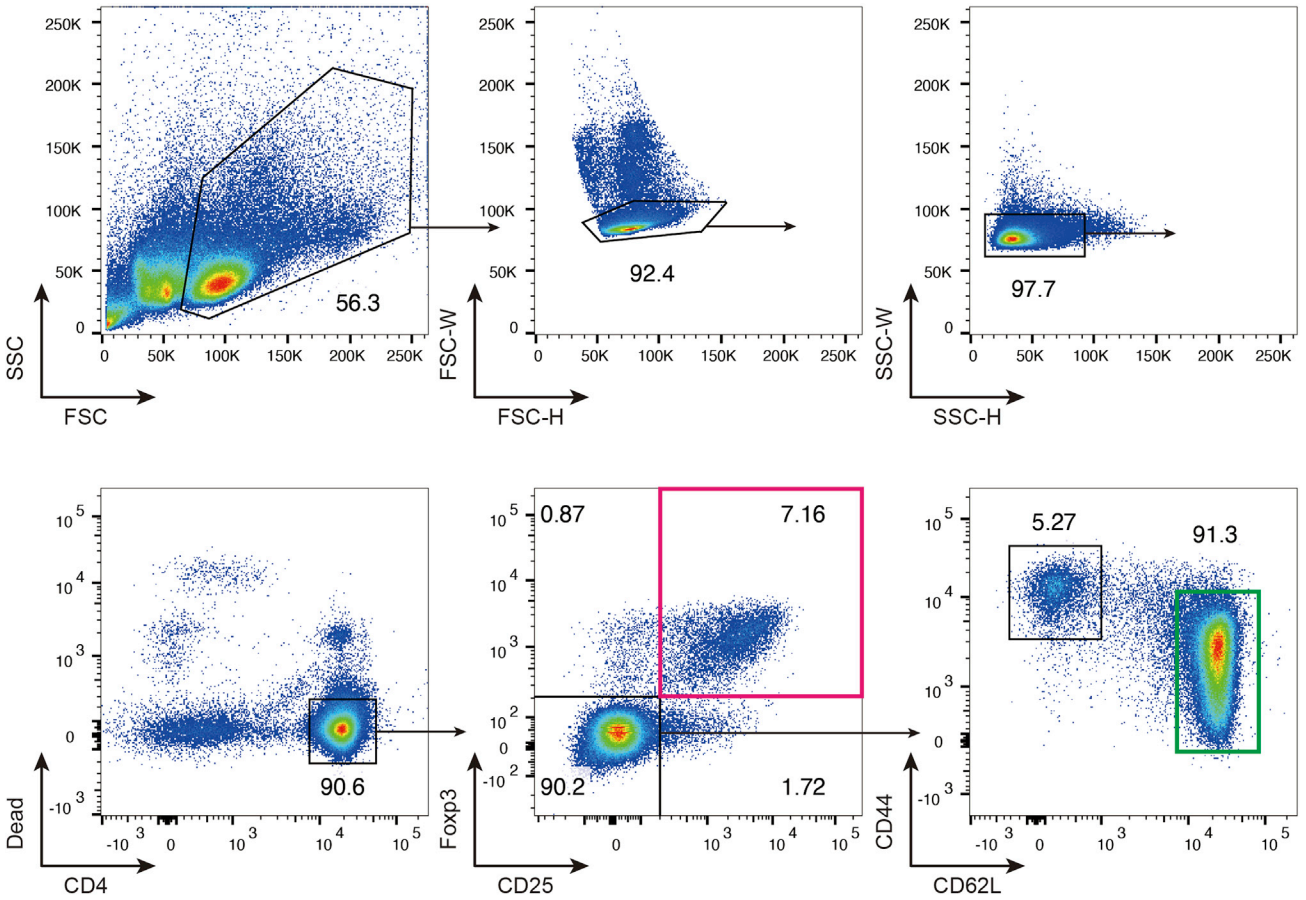

Figure 2. Gating strategy for nTreg and naïve Tconv cells

CD4 enriched lymphocytes from Foxp3-eGFP mice were used as FACS sample. The magenta gate indicates nTreg ($CD25^{+}Foxp3^{+}$), conventional T cells (Tconv; $CD25^{-}Foxp3^{-}$), and the green gate as naïve Tconv ($CD62L^{hi}CD44^{lo}$). Sorted nTreg and naïve Tconv cells were used for the bisulfite sequence.

25. Add 400 μL CIA to the sample tube and vortex well. Centrifuge it at 15,000 × *g* for 5 min at room temperature.
26. Repeat step 23. Add 1 μL Ethachinmate, 40 μL 3 M sodium acetate and 1 mL 100% EtOH to the sample tube. Mix thoroughly by gentle inversion. Centrifuge it at 15,000 × *g* for 20 min at 15°C.
27. Discard the supernatant carefully and add 500 μL 80% EtOH to the sample tube. Centrifuge it at 15,000 × *g* for 15 min at 4°C.
28. Discard the supernatant carefully and dry up pellet for 5 min.
29. Elute the pellet with 20 μL TE buffer.

Note: If you use the fixed cells, please elute the pellet with 400 μL TE buffer, repeat step 21 at 65°C and repeat DNA extraction step again.

Bisulfite conversion

Timing: 2 h

This section describes bisulfite conversion in unmethylated CpG DNA.

The bisulfite base conversion was carried out using the Methyl Easy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures) by following the manufacturer's protocol. If not using

Table 3. Components of bisulfite solution

Reagent	Final concentration	Amount
Sodium bisulfite	6.3 mM	0.66 g
0.45 M NaOH	N/A	1 mL

the kit, please perform the protocol below, we added minor modifications to previous reports (Frommer et al., 1992; Olek et al., 1996; Rakyan et al., 2004; Darst et al., 2010; Harrison and Parle-McDermott, 2011).

Note: We used Zymo-Spin IC Columns as the collecting columns in the alternative protocol.

Note: We set heat blocks at 80°C, 37°C, and 95°C.

30. Prepare the bisulfite solution (Table 3, for 4 samples). Set this solution at 80°C.

Note: 3.5 mM sodium disulfite in 0.45 M NaOH solution is the substitute as the bisulfite solution.

31. Add 2.2 μL 3 M NaOH to 20 μL sample. Set the sample tube for 15 min at 37°C.
32. Add 220 μL bisulfite solution to the sample tube. Set the sample tube for 1 h at 80°C.
33. Add 500 ng salmon sperm DNA solution and 240 μL Buffer PB to the sample tube.
34. Transfer the sample to the column and centrifuge it at 15,000 × *g* for 30 s at room temperature.
35. Discard the flowthrough. Add 200 μL Buffer PE to the column and centrifuge it at 15,000 × *g* for 30 s at room temperature.
36. Repeat step 35.
37. Discard the flowthrough. Set the column on the new 1.5 mL tube and add 50 μL 50 mM NaOH to the column.
38. Set the column for 1 min at room temperature. Centrifuge it at 15,000 × *g* for 30 s at room temperature.
39. Set the sample for 30 min at 95°C for the desulfonation. Add 5 μL 1 M TrisHCl (pH7.5) to the sample.
40. Set the sample on ice. Now, it is ready to use the sample for bisulfite PCR.

Pause point: Able to store the sample at 4°C until next step.

Note: 3 M NaOH will be expired in 4 weeks. Please prepare the solution freshly if possible.

Note: 10 mM CAPS buffer is the substitute as 50 mM NaOH in step 37. In this case, adding 1 M TrisHCl is not necessary.

Note: Insufficient desulfonation leads to failed bisulfite PCR. Desulfonation for 30 min or more is better.

Bisulfite PCR

Timing: 2.5 h

This section describes targeted bisulfite PCR in TSDRs.

We used the primers for the bisulfite PCR (Table 4) from Ohkura et al. (2012).

Table 4. Primers for the bisulfite PCR

Primer name	Sequence (5′-3′)
mFoxp3_CNS2_Fwd	ATT TGA ATT GGA TAT GGT TTG T
mFoxp3_CNS2_Rev	AAC CTT AAA CCC CTC TAA CAT C
mIkzf2_intron3a_Fwd	AGG ATG GTT TTT ATT GAA GGT GAT
mIkzf2_intron3a_Rev	ATA CAC ACC AAA CAA ACA CTA CAC C
mIkzf4_int-last2_Fwd	TAA GAA ATT GGG TGT GGT ATA TGT A
mIkzf4_int-last2_Rev	TTT CCC CTA CTA AAA CTC CTT AAA C
mCtla4_exon2_Fwd	TGG TGT TGG TTA GTA GTT ATG GTG T
mCtla4_exon2_Rev	AAA TTC CAC CTT ACA AAA ATA CAA TC
mIl2ra_int1(2500)_Fwd	TTT TAG AGT TAG AAG ATA GAA GGT ATG GAA
mIl2ra_int1(2500)_Rev	TCC CAA TAC TTA ACA AAA CCA CAT AT
mTnfrsf18_exon5_Fwd	GAG GTG TAG TTG TTA GTT GAG GAT GT
mTnfrsf18_exon5_Rev	AAC CCC TAC TCT CAC CAA AAA TAT AA

Note: If you need some different primers or design these on distinct regions, we recommend using MethPrimer software (Li and Dahiya, 2002).

We used TaKaRa Ex Taq® Hot Start Version (Takara) for bisulfite PCR.

41. Prepare the bisulfite PCR mix (Table 5). Set this mix on ice until PCR.
42. Run the PCR program below (Table 6).
43. Electrophoresis of the PCR products using 2% agarose gel (Figure 3).
44. Cut the aimed band in the gel and transfer the gel pieces to 1.5 mL tube.

Pause point: Able to store the sample at –30°C until next step for 1 month.

Gel extraction

Timing: 30 min

This section describes extraction of bisulfite PCR products from gel pieces.

The DNA extraction from the gel pieces was carried out using QIAEX II Gel Extraction Kit (QIAGEN) by following the manufacturer's protocol.

45. After dissolving and removing the gel and washing with the silica beads (QIAEX II Suspension), elute the pellet with 8 μL TE buffer.

Ligation of PCR products for TA cloning

Timing: 30 min–2 h

Table 5. Components of the bisulfite PCR mix

Reagent	Amount
10× ExTaq buffer	2.0 μL
dNTP mix (2.5 mM each)	1.6 μL
Primer mix (10 μM each)	1.6 μL
ExTaq HS (5 U/μL)	0.5 μL
Water	10.3 μL
Sample	4.0 μL
Total	20 μL

Table 6. Bisulfite PCR program using Ex Taq Hot Start Version

Steps	Temperature	Time	Cycles
Initial denature	98°C	1 min	1 cycle
Denature	98°C	10 s	40 cycles
Annealing	55°C	30 s	
Extension	72°C	45 s	
Final extension	72°C	2 min	1 cycle
Hold	18°C	∞	

This section describes ligation of extracted PCR products into the vector.

The TA cloning was carried out using DynaExpress TA PCR Cloning Kit (BioDynamics). We used the water bathes at 42°C and 37°C.

46. Prepare the vector ligation mix (Table 7) in 1.5 mL tube. Set the tube on ice until a ligation step.
47. Set the tube onto a heat block at 16°C for 30 min–2 h.

DH5α transformation

Timing: 1 h

This section describes transformation of competent cells.

Note: Add 50 μL 0.1 M IPTG and 50 μL 2%(w/v) X-gal to the Kanamycin-added LB plate and spread these solutions well.

48. Add optimized DH5α competent cells solution to the tube. Set the tube on ice for 10 min.

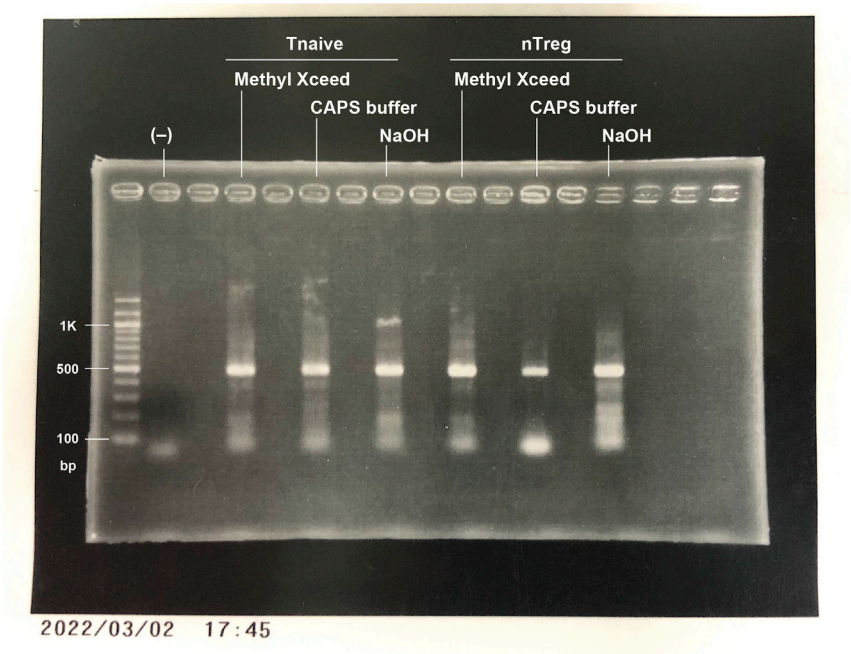

Figure 3. Electrophoresis of bisulfite PCR products in *Foxp3*-CNS2 region of naïve Tconv and nTreg cells
We used the Methyl Easy Xceed Rapid DNA Bisulphite Modification Kit as a commercial bisulfite kit, and the original protocol using two different elution buffers.

Table 7. Component of the ligation mix

Reagent	Amount
2× Ligation buffer	1.2 μL
pTAKN-2 vector (50 ng/μL)	0.2 μL
Ligation mixture	0.2 μL
Sample	0.8 μL

49. Set the tube in the water bath for 45 s at 42°C.
50. Transfer the tube on ice. Add SOC solution (10 times volume of the sample) to the tube.
51. Set the tube in the water bath for 30–45 min at 37°C.
52. Centrifuge the tube at 2,200 × *g* for 3 min at room temperature.
53. Discard the supernatant until 100 μL. Spread the cell suspension gently and well.
54. Put the plate in the incubator (5% CO_2 37°C) for 12–16 h.

Pause point: The colony formed plate can be stored at 4°C until you move to the next step.

Colony PCR

Timing: 4.5 h

This section describes colony PCR after DH5α transformation.

Note: We used illustra TempliPhi DNA Amplification Kit for Rolling Circle Amplification. In addition, we modified the protocol from the manufacturer.

55. Aliquot 2.5 μL Denature buffer into the PCR tube or 96 well PCR plate.
56. Pick up a white colony by the toothpick, then dip the colony into the Denature buffer.
57. Set the sample on the thermal cycler for 3 min at 95°C.
58. Remove the sample from the thermal cycler and add 2.5 μL Premix into it.
59. Set the sample on the thermal cycler for 4 h at 30°C, 10 min at 65°C, and keep it at 25°C.
60. Remove the sample from the thermal cycler.

Pause point: Able to store the sample at 4°C until next step for 24 h.

Big dye

Timing: 2 h

This section describes Big Dye PCR.

Note: We used BigDye™ Terminator v3.1 Cycle Sequencing Kit for analyzing the PCR product-inserted plasmid vector. In addition, we modified the protocol from the manufacturer.

61. Prepare the BigDye mix below (Table 8), and put the mix on ice in dark until the reaction.
62. Run the program below (Table 9) by the thermal cycler.
63. Remove the sample from the thermal cycler.

Pause point: Able to store the sample at 4°C in dark until next step for 24 h.

Note: We performed our original modified protocol here. Please check each setting in your lab.

Table 8. Component of BigDye mix

Reagent	Amount
BigDye™ Terminator 3.1 Ready Reaction Mix (1:16)	2.0 μL
M13 reverse primer (5′-CAG GAA ACA GCT ATG AC-3′)	1.0 μL
Water	1.0 μL
Sample	1.0 μL

Purification of the sequencing reactions

Timing: 10 min

This section describes purification of Big Dye PCR products before the sequencing.

64. Add 1.5 μL CleanSEQ beads and 40 μL 80% ethanol into each well.
65. Vortex and spin down, and set the PCR tube or plate on the magnetic rack for 5 min.
66. Aspirate the supernatant with taking care of the beads.
67. Add 100 μL 80% ethanol into each well.
68. Repeat steps 65 and 66.
69. Dry the beads well. Add 18 μL water into each well.
70. Repeat step 65.
71. Transfer the sample-eluted water to the sequencing plate.

Note: Do not transfer the beads to the sequencing plate for avoiding a sequencing error.

Sequencing

Timing: 1 h

This section describes definition of the TSDR methylation by Sanger sequencing.

Note: We used 3500×L Genetic Analyzer for Sanger sequencing and performed "RapidSeq" mode.

72. Prepare the sample sheet and modify the sequencing setting.
73. Sequence the sample.
74. Analyze the base called sequence data by base analyzing tools.
75. Check the methylation status according to the reference sequence. We show the *Foxp3* CNS2 region as an example below (Table 10).

EXPECTED OUTCOMES

For representative gating strategy of flow cytometry data, see Figure 2. As previous several reports, naturally occurring Treg (nTreg) cells would establish the Treg-specific demethylation in *Foxp3* conserved non-coding sequence (CNS) 2 region as well as other TSDRs such as *Tnfrsf18*,

Table 9. BigDye program

Steps	Temperature	Time	Cycles
Initial denature	96°C	1 min	1 cycle
Denature	96°C	10 s	35–37 cycles
Annealing	50°C	5 s	
Extension	60°C	2 min	
Hold	18°C	∞	

Table 10. CpG position of *Foxp3*-CNS2 region before/after bisulfite conversion (the bold regions are primer-binding regions and under bars show CpG)

Region name	Amplified sequence
Foxp3-CNS2	AACCTTGGGCCCCTCTGGCATCCAAGAAAGACAGAATCGATA GAACTTGGGTTTTGCATGGTAGCAGATGGACGTCACCTACCAC ATCCGCTAGCACCCACATCACCCTACCTGGGCCTATCCGGCTAC AGGATAGACTAGCCACTTCTCGGAACGAAACCTGTGGGGTAGA TTATCTGCCCCCTTCTCTTCCTCCTTGTTGCCGATGAAGCCCAA TGCATCCGGCCGCCATGACGTCAATGGCAGAAAAATCTGGCC AAGTTCAGGTTGTGACAACAGGGCCCAGATGTAGACCCCGAT AGGAAAACATATTCTATGTCCCAGAAACAACCTCCATACAGCTT CTAAGAAACAGTCAAACAGGAACGCCCCAACAGACAGTGCAG GAAGCTGGCTGGCCAGCCCAGCCCTCCAGGTCCCTAG TACCACTAGACAGACCATATCCAATTCAGGT
Bisulfite-converted *Foxp3*-CNS2 (complete demethylation)	AACCTTAAACCCCTCTAACATCCAAAAAAAACAAAATCAATAA AACTTAAATTTTACATAATAACCAAATAAACATCACCTACCACA TCCACTAACACCCACATCACCCTACCTAAACCTATCCAACTAC AAAATAAACTAACCACTTCTCAAAACAAAACCTATAAAATAAA TTATCTACCCCCTTCTCTTCCTCCTTATTACCAATAAAACCCAA TACATCCAACCACCATAACATCAATAACAAAAAAATCTAACCA AATTCAAATTATAACAACAAAACCCAAATATAAACCCCAATAA AAAAACATATTCTATATCCCAAAAACAACCTCCATACAACTTCT AAAAAACAATCAAACAAAAACACCCCAACAAACAATACAAAA AACTAACTAACCAACCCAACCCTCCAAATCCCTAATACCAC TAAACAAACCATATCCAATTCAAAT

Ctla4, and *Ikzf4* (Polansky et al., 2008; Ohkura et al., 2012; Sakaguchi et al., 2020). On the other hand, naïve T conventional (Tconv) cells showed almost completely methylated *Foxp3*-CNS2 region (Figure 4A). Thus, naïve Tconv cells would be a precise control sample for the basal methylation status. Effector Tconv cells maintained fundamentally similar methylation status as naïve Tconv cells (data not shown). In female cells, X-chromosome inactivation controls the activation status on X-chromosomes functionally and structurally (Bacher et al., 2006; Loda et al., 2022). *Foxp3* is an X-linked gene, and regulated by the same mechanism (Nunzio et al., 2009). Analyzing *Foxp3* methylation status, female nTreg cells have theoretically 50% methylated *Foxp3*-CNS2 region (Figure 4B). In the past decade, iTreg cells had been thought as quite unstable in not only Foxp3 expression level but also *Foxp3*-CNS2 demethylation status (Ohkura et al., 2013). However, Mikami et al. developed the procedure to produce stable iTreg cells, based on the *Foxp3*-CNS2 demethylation status (Mikami et al., 2020). In addition, Foxp3 expression can be enhanced by supplementing retinoic acids into culture medium (Mucida et al., 2007). In this paper, we also induced stable iTreg cells following the previous several works using ascorbic acid and Tamibarotene as synthetic retinoic acid (Nair et al., 2016; Yue et al., 2016). Adding ascorbic acid trisodium salt into the culture medium, we confirmed the capacity to confer the *Foxp3*-CNS2 demethylation upon the induced iTreg cells while increased Foxp3 expression by retinoid didn't establish stable *Foxp3*-CNS2 epigenome (Figure 5).

LIMITATIONS

This protocol meets the basic needs for analyzing the potential stability of nTreg and iTreg cells, as well as other Tconv cells in most organs, but there are the small number of Treg cells and CD4+ T cells in particular tissue such as large intestine or adipose tissue. If necessary to analyze such tissue-derived Treg cells, further optimization or pooling cells will be needed to acquire the unbiased results. We confirmed the isolation of genomic DNA and bisulfite conversion at least 1,500 cells in optimized conditions. However, small input of initial cell number sometimes leads PCR bias, so we should take the results carefully. Several reports suggest various *Foxp3*-CNS regions regulates stable Foxp3 expression and functional stability of naturally occurring Treg cells (Zheng et al., 2010; Josefowicz et al., 2012; Kawakami et al., 2021). In this protocol, we showed only *Foxp3*-CNS2 methylation, however it is available to analyze the methylation status in other *Foxp3* genomic regions according to the same procedure.

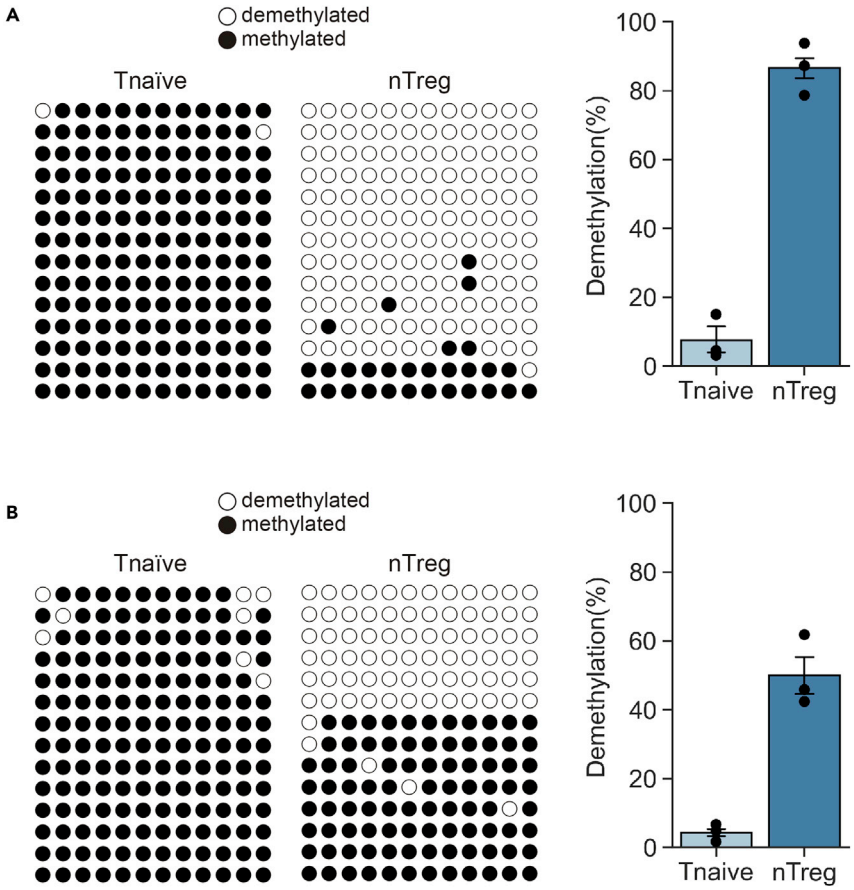

Figure 4. Methylation assay for *ex vivo* naïve Tconv and nTreg cells

(A) Methylation status in *Foxp3*-CNS2 region of naïve Tconv and nTreg cells derived from male Foxp3-eGFP mice. White circles indicate demethylated CpGs and black circles indicate methylated CpGs.

(B) Data derived from female Foxp3-eGFP mice. Barplots and dotplots are represented as mean ± SEM.

TROUBLESHOOTING

Problem 1

Seeing less pellet of fixed cells. (Identified at step 15b of "cell staining and sorting").

Potential solution

Fixed cells are also permeabilized by step a. Permeabilized cells reduced their density and weight, so we can easily lose the cell in steps 15b–15g. Please use centrifuge with high gravity for longer time and wash the cells carefully.

Problem 2

Seeing non-specific stained cell leaking to FITC (eGFP) range. (Identified at step 17 of "cell staining and sorting").

Potential solution

Granulocytes or red blood cells would cause this problem. Generally, granulocytes such as eosinophils contain a bunch of intracellular granules to emit non-specific fluorescence and have large cell bodies. As known, red blood cell sneak into various detector of fluorescence. These cells could be removed by Mouse CD4 T Lymphocyte Enrichment Cocktail (containing clone M1/40 for

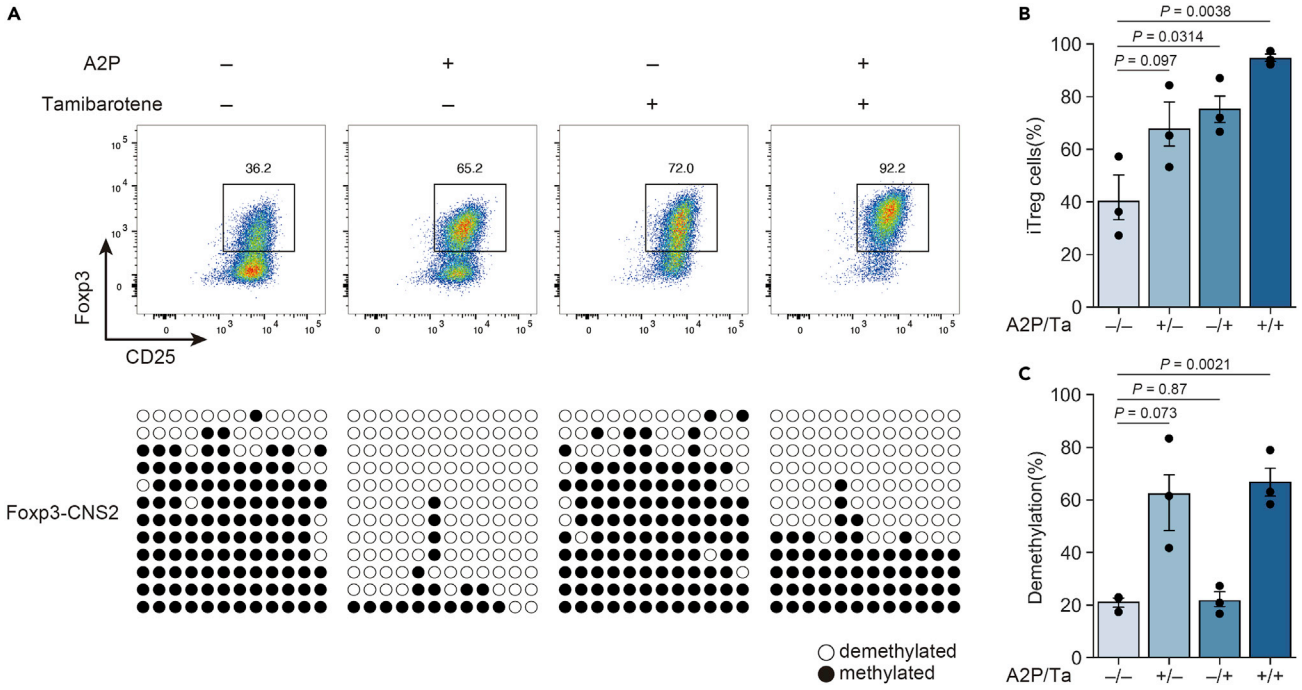

Figure 5. Methylation assay for iTreg cells

(A) Representative data of FACS plots of day 3 induced iTreg cells and *Foxp3*-CNS2 methylation status with adding A2P as ascorbic acid or Tamibarotene as synthetic retinoid to iTreg inducing culture medium. White circles indicate demethylated CpGs and black circles indicate methylated CpGs.

(B) iTreg induction rate in each condition adding A2P or Tamibarotene.

(C) *Foxp3*-CNS2 methylation status in each condition. Barplots and dotplots are represented as mean ± SEM.

granulocytes and Ter119 for red blood cells) in this protocol. Please reduce the size of P1 gating or add increased amount of this cocktail for effective negative selection.

Problem 3

Weak or no band in electrophoresis. (Identified at step 43 of "bisulfite PCR").

Potential solution

There are various possibilities. If you start this procedure with more than 10,000 cells, you can see the pellets after step 18. If not, you should increase input of initial cell number, check the step in gDNA extraction, or increase input of sample into bisulfite PCR. Insufficient bisulfite conversion or desulfonation would lead incomplete annealing of bisulfite PCR primers. Please inspect bisulfite conversion steps. For example, you should treat sample with bisulfite solution for longer time in step 32.

Problem 4

Few or no white colony in TA cloning. (Identified at step 56 of "colony PCR").

Potential solution

Insufficient PCR products or ligation are supposed. Please check the procedure in gel extraction and ligating time. If impossible to see any colony, please check steps in DH5α transformation and optimize preculture and overnight culture, for instance, to do preculture for longer time to enable DH5α to recover from heat shock.

Problem 5

Seeing a lot of guanines in the analyzing step. (Identified at step 75 of "sequencing").

Potential solution

If impossible to get bisulfite-converted data like Table 10, insufficient bisulfite conversion is possible. Please check bisulfite conversion steps. For example, you should treat sample with bisulfite solution for longer time in step 32.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Masaya Arai (marai@ifrec.osaka-u.ac.jp).

Materials availability

This study did not generate any unique reagents.

Data and code availability

This protocol did not in itself generate any data or code availability.

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AUTHOR CONTRIBUTIONS

M.A. outlined and optimized protocol methods, designed the project, performed the experiments, and wrote the manuscript with contributions from all authors. A.F., R.M., Y.N., and Z.C. tested protocol and generated data for write-up. M.A. and S.S. supervised this protocol paper. All authors were involved in manuscript writing and generation.

DECLARATION OF INTERESTS

The authors declare no competing interests associated with this manuscript.

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